EXERCISE- INDUCED EXPRESSION OF MYOCARDIAL HSP72: MECHANISMS OF AGE RELATED ATTENUATION

By

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Chairman: Scott K. Powers
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Recent investigations clearly indicate that induction of heat shock proteins (HSPs), HSP72 in particular, has been associated with myocardial protection. As a non-toxic stimulus, exercise training has been shown to increase HSP72 levels in variety of tissues including myocardium. Since we demonstrated a decreased induction of HSP72 in aged myocardium following endurance exercise, it was of interest to determine the mechanism underlying this diminished HSP response. Young/adult (5-6 months old) and aged (23-24 months old) Fischer 344 rats were randomly divided into control, exercise training, and heat shock groups. Exercise training regime consisted of 2 consecutive days (60 min/day) of running on a motorized treadmill at about 70% of $VO_{2\,max}$. Heat shock was comprised of 15 minutes whole body heat shock at 41.2°C. At selected times following heat stress and exercise training, HSP72, HSP73, and heat shock transcription factor (HSF1) protein content were analyzed by western blotting. The binding of heat shock transcription factor (HSF1) to the promoter region of the HSP72 gene was evaluated by in vitro gel mobility shift assays. Hearts from control, heat stress, and exercise training groups expressed similar levels of HSP73. On the other hand, HSP72 levels in both exercise training and heat stress groups were significantly higher than their age matched controls ($p<0.05$). Both exercise training and heat stress resulted in activation of heat shock transcription factor as we demonstrated shift in the gel mobility shift assays. HSP72 induction was attenuated in the hearts of the aged animals compared to young/adult ($p<0.05$). The attenuation of the HSP72 response in old animals was not due to reduced level of HSF1 expression or less activation of HSF1 in old animals since neither the level of HSF1 nor the binding of HSF1 to DNA was different among groups ($p>0.05$). These results demonstrated that aging is associated with diminished HSP72 response in the myocardium after exercise training. Since the level of HSF and the activation of transcription activation factor did not differ between age groups, mechanisms other than the level of HSF1 or activation of HSF1 are responsible for this impairment. Although it is unknown if this exercise-induced increase in myocardial HSP72 in old animals is sufficient to provide protection against myocardial ischemia-reperfusion injury, the inability of older individuals to respond to acute stress may be partly explained by an inability to mount the HSP response, a key defense mechanism in the cells and tissues of individuals.

INTRODUCTION

Aging is a multifactorial process resulting in damage to molecules, cells, and tissues that exceeds the capacity of the organism to adapt and/or to repair the damage (112). Cells have evolved complex genetic systems to detect specific forms of stress and activate the expression of genes whose products increase the resistance of the cell to further stress and/or to initiate the processes of tissue regeneration. Unfortunately, the expression of many of these genes is attenuated in aging (134). As a consequence, cellular responsiveness to stress is reduced with advancing age.

One of the best understood cellular responses to stress, occurring in organisms ranging from bacteria to humans, is the induction of heat shock proteins (HSPs) (96,98). HSPs are the products of highly conserved genes that are rapidly transcribed and translated in cells exposed to variety of stresses, including heat, ischemia, heavy metals, amino acid analogs, oxidants, transition metals, ethanol, and metabolic poisons such as arsenite and azide (40,98,167,168,171). Recent investigations have revealed that HSPs of the 70 kDa family are important in protecting cells from a variety of stresses including myocardial ischemia-reperfusion (I-R) injury (38,85,118,174). Specifically, the synthesis of the inducible member of 70 kDa HSP family, HSP72, enhances recovery from acute cellular injury and protects the cell from subsequent injury. Indeed, it is well established that heat stress-induced increases in myocardial HSP72 result in reduced myocardial damage following I-R in young animals (25-27).

Regular physical exercise has long been regarded as an important component of healthy lifestyle. Exercise training generates many metabolic changes in both skeletal and cardiac myocytes, including the generation of reactive oxygen species and lactic acid, energy depletion, and calcium ion release from the sarcoplasmic reticulum; each of these metabolic changes are known to induce HSP70 independent from hyperthermia (146). In this respect, we and others have demonstrated that both long-term (10-12 weeks) and acute (3-5 days) endurance exercise training elevates myocardial HSP72 and this increase is associated with a significant reduction in I-R induced myocardial injury and improved myocardial recovery in young animals (32,34,109,138).

Recent evidence indicates that mammalian aging is associated with a decreased ability of cells to express HSP72 in response to heat stress. Specifically, studies done in both tissues and primary cell cultures derived from old animals indicate an attenuated heat shock response (10,12,43,62,64,103). Further, recent experiments in our laboratory indicates that aging is associated with a decreased exercise-induced expression of myocardial HSP72. At present, the mechanism to explain the age-related decline in myocardial HSP72 expression is unknown. Therefore, the objective of these experiments was to determine if the age-related reduction in exercise-induced expression of myocardial HSP72 is due to decreased activation of the HSP72 gene. To address this issue, we will perform studies using intact animals and employ the tools of molecular biology (e.g., Gel mobility shift assay, and Western analysis).

Significance

Heart disease is the most prevalent chronic disease in the United States, accounting for greater than one million heart attacks per year (4,142). Since the heart is composed primarily of post-mitotic cells, prevention of cell loss after ischemia is paramount to myocardial functional recovery following exposure to an I-R insult. Aged hearts are much more vulnerable to acute cardiovascular stress, and more susceptible to I-R injury than are young hearts (74,91,120). The inability of older individuals to respond to acute stress may be explained, in part, by an inability to mount the heat shock response, a key defense mechanism in the cells and tissues of individuals (112). Understanding the mechanism underlying the attenuated HSP72 response in the aged heart is critical to developing protective strategies that result in a myocardium that is better able to resist an I-R insult. Therefore, additional research involving the mechanisms of attenuating HSP72 response in aged hearts is clearly warranted. These experiments will improve our understanding of the mechanisms responsible for the age-related difference in exercise-induced expression of myocardial HSP72. Further, this work could lead to important clinical benefits in the management of heart disease or other disorders that might predispose the aging patient to myocardial injury.

Research Hypotheses

Mammalian aging is associated with an alteration in the ability of cells to express HSP72 in response to acute stress (60,61,63,64,100,101,103). The present study was designed to determine whether the age-related reduction in exercise-induced myocardial HSP72 expression is due to reduced activation of the HSP72 gene. Following acute exercise or heat stress, the myocardium of aged animals was compared to myocardium from adult animals to answer the following questions:

Question 1: Is aging associated with a reduction in myocardial levels of heat shock transcription factor 1 (HSF1)?

Hypothesis 1: Compared to young adult animals, the hearts of senescent animals contain lower levels of HSF1.

Question 2: Is the age-dependent reduction in the induction of myocardial HSP72 in response to exercise training due to less activation of heat shock transcription factor I (less binding of HSF1 to the promoter region of the HSP 72 gene)?

Hypothesis 2: The age-dependent reduction in the expression of cardiac HSP72 in response to exercise training is due to reduced HSF1 binding to the promoter region of the HSP 72 gene and reduced gene activation.

Hypothesis Justification

When subjected to heat stress, cells from aged animals demonstrate a diminished stress response; a reduced accumulation of HSP70 mRNA and protein after heat stress has been reported in aged rat liver cells (60,64) as well as human diploid fibroblasts (99,103). Heat stress in intact animals also results in a diminished heat shock response in the aged myocardium (108). Further, preliminary experiments in our laboratory indicate that compared to young animals, senescence rats accumulate less cardiac HSP72 following exercise following exercise training. A potential mechanism to explain the age related decline in the exercise-induced myocardial HSP72 levels could be the reduced basal level of HSF1 in old hearts.

HSF1 is a heat hock transcription factor that binds to a specific DNA sequence on the HSP gene, called the heat shock element (HSE), to start transcription. Therefore decreased level of HSF1 in aging could account decreased level of HSP72 expression after stress. In hepatocytes isolated from heat stressed young adult and old rats, Heydari et al. reported a 40-50% decrease in HSF-DNA binding activity, HSP72 mRNA, and HSP72 protein levels in senescent liver cells (64). Similar results have been reported in the heart. Using a whole body heat stress model, Locke and Tanguay examined the expression of myocardial HSP72 in the hearts of both young adult and senescent rats (108). In response to heat stress, aging was associated with a diminished level of HSF1-HSE binding, reduced HSP72 mRNA, and a lower HSP72 protein concentration in the myocardium.

Again, preliminary studies in our laboratory indicate that compared to young adult animals, senescent animals accumulate less myocardial HSP72 following 10 weeks of endurance exercise training (500% vs. 300 % increase in old myocardium). The mechanism responsible for this attenuated induction of HSP72 after exercise is unknown. Based on aforementioned studies performed in both intact tissues and isolated cells derived from young and old animals, it seems reasonable to hypothesize that there is a less binding of HSF1 to HSE in aging myocardium after exercise. Our proposed experiments will test this postulate.

REVIEW OF RELATED LITERATURE

Introduction

The response to environmental and physiological stress includes a highly ordered set of events that is often represented by rapid changes in gene expression followed by the synthesis of proteins involved in adaptation to stress (122). The types of genes induced are dependent on the nature of stress. Although there is an overlap in the responses, heat stress induces a particular sets of genes, while oxidative stress and DNA damage others (144).

Advanced age is associated with impairment in most physiological functions, particularly in the ability to maintain homeostasis during environmental and physiological stress (152). Although the biochemical and molecular bases of these age-related detriment are unknown, functional decrements at the cellular, tissue and organ levels contribute to the increased morbidity and mortality associated with age.
Recent studies have provided evidence that in response to a variety of stresses, cellular system initiate the preferential synthesis of a group of proteins, termed "heat shock proteins" (reviewed in 123). The physiological stress created by exercise training is also capable of inducing heat shock genes in mammals (reviewed in 104). In contrast, heat shock genes are poorly induced in aged animals (11,43,108,175) or cell cultures undergoing replicative senescence (21,99,100,103,110). The purpose of this review is to

provide a brief overview of our current understanding of the expression and biological function of heat shock proteins. Further, this review includes a discussion of the effects of aging and exercise on expression of important myocardial heat shock proteins.

The Heat Shock Proteins

General Features

One of the best understood cellular responses to stress, occurring in organisms ranging from bacteria to humans, is the induction of groups of highly conserved stress (39,40, 97,98,149). These proteins increase rapidly when the cells are exposed to a variety of environmental stresses (167). Since the most studied stress is heat shock, these proteins are often called heat shock proteins (HSPs) (145).

HSPs have been implicated in a number of cardiovascular research areas including ischemia, hypertrophy, aging and atherosclerosis. For example, recent investigations have revealed that overexpression of HSP 72, the major inducible protein in the 70 kDa subfamily, can protect the myocardium from ischemic and hypoxic injury (15,71).

History and Nomenclature

The heat shock response, defined as the rapid induction of HSPs, was first identified in 1962 by Ritossa in *Drosophila busckii* (145). Subsequently, by analyzing newly synthesized proteins in *Drsosphilia* larvae incubated at 37.5°C for 20 min, Tissieres et al. discovered several major heat shock proteins (162). This initial observation led to the characterization of a rapidly increasing number of isoforms as members of HSP families.

The synthesis of HSPs is induced not only by hyperthermia but also by a variety of other stresses (e.g., hypoxia, changes in pH levels, oxidants, ischemia/reperfusion, transition metals, aminoacid analogs) (40,65,92,98,116,118,167,168,171). Hence, this generalized response to perturbations in the normal living environment of cells is also referred to as the " stress response", and accordingly the proteins induced are termed the "stress proteins" (40). Recent studies have revealed that physiological stress created by chronic treadmill running or physical restraint also results in rapid increase in the expression of HSPs in rodents (12,32-34,105-107,138,146).

Classification of Heat Shock Proteins

Heat shock proteins, from prokaryotes to eukaryotes, represents one of the best known conserved groups of proteins. The induction of these proteins after exposure to heat or to an amino acid analogue is also very similar in different mammalian cell lines (170). Based on their migration during SDS-PAGE, five main groups of heat shock proteins are recognized: 1) small HSPs, 8 to 32 kDa; 2) 50-60 kDa HSPs; 3) 70 kDa HSPs; 4) 90 kDa HSPs; and 5) 100kDa plus HSPs (85). Although the number of HSPs varies from organism to organism and even between cell types in an organism, all organisms express 65 to 78 kDa HSPs which belong to the HSP70 family (97). Since the focus of our proposed experiments is on the HSP72, only a brief overview of other families of HSPs will be provided.

Small heat shock proteins

There are five different prominent members of the small HSPs family: ubiquitin, HSP20, HSP27, ∝B-crystallin, and HSP32. Ubiquitin is an ~8-kDa, highly conserved protein and serves a role in both chromatin structure and in protein degradation events.

Many intracellular proteins that are to be degraded are first covalently modified by the ubiquitin. Presumably after heat shock and other forms of stress, the resulting increases in ubiquitin levels facilitate the targeting and removal of denatured proteins (167). Indeed, an increase in the amount of ubiquitin-conjugated proteins after heat shock has been reported (19). Basically, denatured proteins, conjugated to ubiquitin, are targeted for degradation via the nonlysosomal proteolytic pathway.

HSP20 was recently isolated from rat and human skeletal muscle and its amino acid sequence is closely related to both HSP27, and ∝B-crystallin. Limited information about the biological function of HSP20 is currently available.

∝B-crystallin, a 22-kDa protein, belongs to a family of crystallins found in vertebrate lenses. In addition to lens tissue, ∝B-crystallin it is expressed in tissues that possess high levels of mitochondria such as cardiac and type I and IIa skeletal myofibers (131). It functions as a molecular chaperone and has been shown, in vitro, to prevent aggregation of denatured proteins in response to stress and facilitate protein folding upon removal of stress.

HSP27 is also designated as HSP25 or HSP28. The constitutive expression of HSP27 appears to be growth regulated with the relative amounts of the protein increasing as cells reach the stationary phase (167). HSP27 is known to be the main phosphoprotein expressed in a variety of cells in response to cell stimuli, and phosphorylated HSP27 confers resistance against actin fragmentation (70). Similar to ∝B-crystallin, HSP27 has been shown to occur in relatively high concentrations in heart tissues (29,66). Studies on the localization of HSP27 strongly point to a regulatory role in muscle contraction (150).

HSP32 is an inducible isozyme of the heme oxygenase system and is referred to as heme oxygenase 1 (HO-1). Both HO-1 and the constitutive isozyme HO-2, generate the cellular messenger carbon monoxide (CO) in mammalian cells. The HSP32 gene is strongly induced by oxidizing agents (81) and has been shown to be induced by oxygen free radicals (80,114,164).

The 50-60-kDa heat shock proteins

The members of the 50-60 kDa HSPs family are often labelled as "molecular chaperones" (42). These proteins belong to a family of related molecular chaperones containing two distinct subfamilies; the GroE subfamily found in the eubacteria, plastids, and mitochondria, and the TCP-1 subfamily found in the eukaryotic cytosol and the archebacteria. Both chaperone subfamilies assist the correct folding of newly synthesized polypeptides in the cytosol of prokaryotes and eukaryotes (41,49).

The 90-kDa heat shock proteins

HSP90 is the most abundant constitutively expressed stress protein in eukaryotic cytosol. HSP90 is bound to a wide range of proteins such as viral tyrosine kinases and steroid hormone receptors (139). HSP90 is believed to play a role in assisting the folding of newly synthesized polypeptides in vivo (169).

The 100kDa plus heat shock proteins

Both HSP100 and HSP110 are normal constituents of mammalian cells and are generally expressed at low levels (159). Both proteins are inducible upon heat shock. Despite their similarities to other stress proteins, their biochemical function in the cell is not well defined.

The 70-kDa heat shock proteins

The 70 kDa-HSPs are very abundant proteins accounting for as much as 1-2% of all cellular protein (59). HSP70 genes were first visualized as puffs on chromosomes in salivary glands of Drosophilia upon exposure to high temperature or (145). Eukaryotic 70kDa-HSP genes are ancestrally related to *Escherichia coli* dna K, and encode a large multigene family of proteins. It appears that at least four groups of genes make up the HSP70 family. The most prominent members of HSP70 family, HSP72 and HSP73, are located within both cytoplasm and nucleus. Two other major forms of the HSP70 are the mitochondrial, and sarcoplasmic glucose-regulated proteins (GRP); these are referred to as GRP75 and GRP78, respectively (128,167). GRPs are induced by glucose starvation.

HSP73 which is constituively synthesized in all mammalian cells is commonly referred to as the HSP70 cognate, or HSC73. The synthesis of HSP72 is usually restricted to the cell experiencing stress and therefore is often referred to as the inducible form of HSP70. These two 70kDa-HSP, exhibit extremely high sequence homology (~95%) and similar biochemical properties.

Regulation of Heat Shock Gene Transcription

Regulation of HSP genes is mediated by heat shock/stress transcription factor (HSF) (135,176). In unstressed cells, HSF is a monomer without DNA binding ability. In response to heat shock and other cellular stresses, HSF becomes activated by acquiring an oligomeric state (176). Upon activation, HSF binds to a specific modular sequence elements within HSP gene promoters (4,178), referred to as the heat shock element (HSE). This binding and subsequent activation results in elevated transcription of genes encoding HSPs (45,121,123,136,177). The activation of the DNA binding form of HSF can be detected within minutes of temperature elevation (125). The kinetics and magnitude of DNA binding activity during heat shock are proportional to the transcriptional response (2). In mammalian cells, activation of HSF involves a number of inducible events including oligomerization, acquisition of DNA binding, translocation into the nucleus, and increase in transcriptional activity (22,148,155).

It has been speculated that eukaryotic cells contain a regulatory protein that could hinder the DNA-binding of HSF activity (22). HSP72, shown to interact with HSF1 in eukaryotes, has been implicated as a candidate for this regulatory protein (1). Several observations from Morimoto's lab have lead to a model for HSF activation. According to this model, under non-stressful conditions, HSF is maintained in a non-DNA binding state through interactions with HSP 72. During heat shock or any other forms of stress, the appearance of denatured proteins creates a large pool of new protein substrates that competes with HSF for association with HSP72. Therefore, the appearance of denatured proteins results in the release of HSF from HSP72; the released HSF oligomerizes and then binds to DNA. The activation of HSF-DNA binding leads to an elevation of transcription and synthesis of HSP72 (123,125).

HSF Functional Domains and Family Members

Whereas yeast and fruit flies appear to have only a single type of HSF, higher eukaryotes encode an HSF multi-gene family. All heat shock transcription factors from eukaryotes have a number of common features. First, the amino-terminal localized DNA binding domain is approximately 100 amino acids in length. Further, hydrophobic repeats of the oligomerization domain exist and appear to be essential for trimer formation (136,155). Finally, C terminal hydrophobic repeats occur which participate in intramolecular negative regulation (83,84).

Two HSFs have been identified in rodents; HSF1 and HSF2. Although HSF1 and HSF2 are structurally related and 40 % identical in overall amino acid sequence, they are distinct by regulatory and functional criteria. HSF1 is the activator of heat shock gene transcription in response to elevated temperature, heavy metals, amino acid analogues and oxidative stress, whereas HSF2 is activated in response to signals during early development and differentiation (9, 75, 123,177). Thus, the signals that activate the DNA binding properties of each factor are specific. Activation of HSF1 is a complex multi-step process involving an oligomerization from inert monomer to active trimer, acquisition of DNA binding ability, phosphorylation, and nuclear localization (124). In contrast, the acquisition of HSF2-DNA binding activity is accompanied by the transition from an inert dimer to a trimer (89,124). In addition to HSF1 and HSF2, HSF3 and HSF4 have been identified in chickens and humans, respectively (129,130).

Biochemical Role of 70-kDa HSPs

Our understanding of the physiological roles of the HSP70 family of proteins in eukaryotes is growing. Numerous studies have shown that HSP72 and HSP73 interact with variety of proteins, play a role in protein maturation, protein transport, proteolysis, and are involved in cellular protection against a variety of (see 51,104,167 for reviews).

The life cycle of eukaryotic proteins include their synthesis on ribosomes, sorting to a specific subcellular compartment, folding to an active conformation and finally its turnover. The optimal functioning and maintenance of any cell is explicitly dependent upon the presence of correctly folded proteins; therefore, chaperoning function of HSPs and particularly HSP72 is very important (53). As a key component of the chaperone machine in eukaryotes, HSP72 associates with nascent chains on ribosomes to control the folding of newly synthesized proteins (8,46). HSP72 is involved in stabilizing nascent polypeptides prior to their translocation into the endoplasmic reticulum and mitochondria (35). The interaction of HSP70 with unfolded proteins is carried out via an ATP dependent mechanism that occurs as follows: 1) HSP70-ADP complex binds to an unfolded polypeptide; 2) after prefolding of the bound polypeptide, a new molecule of ATP replaces ADP; 3) ATP hydrolysis or exchange of ADP for ATP occurs and HSP70-ADP complex is released from prefolded protein and binds to new substrate (9,75,79,133).

Besides the regulation of polypeptide functioning, post-translational protein sorting and translocation into mitochondria and endoplasmic reticulum requires the action of HSP70 family members (see 16 for review). In this regard, it has been reported that there is more than one mechanism by which HSP70 proteins facilitates the transfer of proteins across the intracellular membranes (151, 160).

HSP70 proteins are also involved in proteolysis. Mammalian cytosolic HSP70 proteins recognize specific sequences in damaged proteins and help target them into lysosomes for degradation (20, 36). The role of HSPs in proteolysis may be to prevent aggregation and maintain the accessibility of substrate proteins to the proteolytic system rather than to actively facilitate protein degradation (119).

HSP72 and Cellular Protection Against Heat Stress

The first evidence that HSP72 was a cytoprotective protein was largely correlative in nature. However, many recent studies using both transfection of the HSP72 gene into cultured cells and transgenic mice models have provided direct evidence that HSP72 provides cellular protection against a wide variety of stresses (5,15,48,72,82,95,102, 111,117,137). One of the most studied stresses is heat stress.

Early experiments demonstrated that when cells were subjected to sub-lethal heat stress (i.e., sub-lethal but sufficient to induce the expression of HSP72), the cells were able to survive a second more rigorous (i.e., lethal) heat stress (50,56,96). It is now clear that this acquired thermotolerance phenomenon is dependent upon the increased expression of the heat shock proteins (57,96). This thermoprotection is also conferred when HSP's are induced by stresses other than heat (93). Evidence exists that the amount of thermal protection is directly related to the concentration of cellular HSP72 (158). Indeed, There is a strong correlation between cellular HSP72 content and the logarithm of cell survival in Chinese hamster HA-1 cells after thermal stress (94).

Direct evidence that HSP72 provides cellular protection against heat stress has been provided by several investigators. For example, Angelidis (5) has demonstrated an acquired resistance to elevated temperature in cell lines by increasing the basal levels of HSP72 via transfection of the cells with a plasmid construct consisting of the human HSP72 gene (5). Similarly, transfection of rat fibroblasts with constitutively expressed recombinant human HSP72 encoding gene, or transfection of a rat myocyte cell line with the human HSP72 gene under control of ß-actin promoter resulted in protection against lethal heat stress (55,95). Increases in cellular levels of HSP72 following heat exposure parallel the increased protection against nuclear protein aggregation (157). In cells injected with HSP72 antibodies, heat-induced nuclear translocation of HSP72 is blocked (49) and cells quickly died after exposure to a brief heat shock treatment (49,143).

The Cardiac Cell and HSP72

Cardiac and skeletal muscle myocytes are capable of responding to various types of stressful stimuli by the induction of HSPs (47,73). Indeed, it well established that heat shock proteins are readily synthesized in cardiac cells in response to a variety of stressful stimuli. In an early study, the core body temperature of rodents was elevated to 42°C-42.5°C for 15 minutes, and proteins were labeled with [35S]-methionine during a 2 hour recovery period (28). Examination of labeled proteins revealed that there was a depression in overall protein synthesis, but the synthesis of two 70-kDa proteins was increased. These highly induced proteins were referred to a 71-kDa protein which is now known as HSP72; the other moderately-induced protein was later identified to by HSP73. These early findings were confirmed the following year as the synthesis of similar protein was reported in the rat heart after aortic banding or heat stress (52).

Ischemia and/or reperfusion have also been shown to induce HSP72 gene expression in heart tissue (115,116). For example, Dillmann et al. have shown an increase in the HSP72 gene expression following 6 hours of left anterior descending artery occlusion in the dog heart (37). In the same year, hypoxic hypoxia produced by hypoxia decompression was also found to increase the synthesis of HSP in vivo in rodent cardiac tissue (69). In 1991, Knowlton et al. found that as little as 5 minutes of ischemia increases the HSP72 mRNA in the ischemic zone in rabbit heart (86). Repetitive short duration ischemia and reperfusion (four sets of 5min ischemia and 5 min reperfusion) increases myocardial HSP72 content as early as 2 hours, with maximal accumulation after occurring 24 hours following the last insult (29,111,161).

Mechanical overload has also been shown to induce HSP72 in myocardium. Three different experimental models have been used to study this form of cardiac stress: 1) in vivo hearts; 2) isolated perfused hearts; and 3) cultured myocytes. In an *in vivo* model, HSP72 is induced by aortic banding (52,76,154). Thirty minutes after initiating aorta banding, HSP72 mRNA begins to appear in the heart (76) and within 3 hours HSP72 induction was detected in rat myocardium (154). In an *in vitro* model, elevation of perfusion pressure has also been reported to increase HSP68 and genes encoding for proto-oncogenes (c-fos and c-myc) (30,31).

Recent evidence indicates that muscular exercise induces HSPs in skeletal muscles, liver, spleen, adrenal, brain and heart of the rats (32,33,34,107,109,138,146). Since our proposed experiments involved exercise-induced HSP72 gene regulation in cardiac myocytes, exercise induced HSP72 expression will be discussed in more detail later in the review.

HSP72 and Myocardial Protection

A large amount of evidence indicates that a high level of myocardial HSP72 provides protection against an ischemia-reperfusion (I-R) insult. Indeed, numerous studies have shown that induction of myocardial HSP72 by whole animal heat stress confers significantly improved myocardial salvage following coronary occlusion and reperfusion *in vivo* (27,32,39,40,111) as well as enhanced post-ischemic contractile recovery in an isolated perfused heart model (25,165). An illustration of this protection follows.

In a recent study, heat stressed animals and non-heat stressed controls were subjected to 35 minutes of left coronary artery occlusion and 120 minutes of reperfusion. Twenty-four hours following heat stress, analysis of hearts from heat stressed animals revealed a significant increase in HSP72. Further, the infarct size was significantly reduced in heat stressed rats compared with controls (40).

Exercise-Induced HSP72 Expression and Myocardial Protection

Regular physical exercise has long been regarded as an important component of a healthy lifestyle. A number of myocardial benefits have been attributed to exercise, yet the exact mechanism (s) for exercise-induced protection to the myocardium continues to be debated (13). Nonetheless, there is growing evidence that much of the exercise training-induced myocardial protection against an I-R insult is linked to increases in myocardial levels of HSP72 (32, 34,106,107,109,138).

The mechanistic link between exercise and cellular expression of HSP72 is unclear. However, there are several possible mechanisms whereby exercise can induce HSP production: 1) increases in tissue temperatures (105); 2) elevated oxidative stress (146); 3) progressive depletion of glucose and glycogen stores; 4) increase in cytosolic calcium levels; and 5) decrease in intracellular pH. Although the exact component of exercise responsible for the induction of HSP72 remains unknown, it is plausible that two or more aforementioned factors may be acting in a synergistic manner (104). As a non-toxic stress, exercise is an interesting tool by which to study the regulation of HSP gene expression.

Similar to heat stress, both acute and chronic running exercise can induce HSP72 in a variety of tissues including myocardium (32,34,87,105,107, 109,138,146,153). In an early study, one bout of exercise (treadmill running to exhaustion) has been shown to increase HSP72mRNA and HSP72 protein in skeletal muscle, heart, and liver in rats. Immediately following exercise, the levels of two HSP72 mRNA species (2.9 kDa. and 3.3 kDa.) increased in myocardium (146).

Locke et al. performed the experiment to associate exercise-induced HSP72 expression with myocardial protection (109). In that study, hearts from control, heat stressed, and exercised (3 consecutive days treadmill running) rats were removed and placed in a *in vitro* Langendorff apparatus; the hearts were then subjected to 30 min of global ischemia followed by 30 min of reperfusion. The results revealed an improved functional recovery (i.e., indicated by maximal rate of contraction) in both the exercised and heat stressed animals compared to controls. Analysis of myocardial levels of HSP72 revealed a large increase in HSP72 in both the exercise and heat stressed animals.

Our laboratory has performed a series of experiments to elucidate the effects of both acute and chronic exercise on I-R in rat myocardium. We hypothesized that elevation of HSP72 after exercise is associated with myocardial protection against I-R. In two separate experiments, we observed that chronic endurance training (10-12 weeks of treadmill running) resulted in about 500% increase in HSP72 in the rat myocardium (32,138). This increased level of HSP72 was associated with protection against myocardial lipid peroxidation (32), and better maintenance of contractile performance (138) after *in vivo* I-R

In another set of experiments, we examined the relationship between daily exercise training duration of myocardial accumulation of HSP72. In these experiments, three groups of rats were trained 4 days/week (10 weeks) on a motorized treadmill. Training intensities of each group were identical (i.e. 75% of VO2max), while daily exercise durations were varied (30, 60, and 90 minutes/day). Analysis of myocardial levels of HSP72 revealed a step-wise increase in HSP72 levels with the increase in daily exercise duration (unpublished observation).

In another study, we tested the hypothesis that 3-5 consecutive days of exercise is sufficient to elevate myocardial HSP72 expression and protect the myocardium against *in vivo* I-R injury. Animals were assigned to one of four experimental groups: 1) 3 days consecutive exercise (60 min/day); 2) 5 days consecutive exercise (60 minutes/day); and 3) whole body heat stress (15min at 42°C), and 4) sedentary control group. Compared to control, both 3 and 5 days of exercise resulted in elevated myocardial HSP72 levels (34). Twenty-four hours after the last exercise training bout, the animals were exposed to an *in* vivo myocardial I-R insult (30 minutes ischemia followed by 30 minutes reperfusion). During the I-R protocol, left ventricular peak systolic pressure and maximal rate of contraction (+dP/dt) and relaxation (-dP/dt) were recorded via intra-ventricular catheter. Compared to controls, hearts from both exercise training groups demonstrated significantly higher peak systolic pressure and ±dP/dt during both ischemia and reperfusion *in vivo* (34).

Aging and Expression of Heat Shock Proteins

In the unstressed state, aged and young/adult cells express similar levels of HSP 72 (64,108,132). However, when subjected to heat stress, aged cells exhibit a blunted rate of expression. Indeed, strong evidence exists that aging is associated with a decreased expression of HSP 72 in rat brain, liver, skin, and adrenal cells (12,64). and in certain human cell lines (21,103). Recent studies also indicate that there is a diminished expression of HSP 72 in aged rat hearts after both ischemia (132) and heat stress (88). Together, these data indicate that aging is associated with a reduced ability to express HSP72 in response to stress. However, it is unknown if old myocardium has a blunted HSPs response following exercise training. Our experiments designed to answer this question.

The decreased ability to express HSP 72 in aged cells could occur at the cellular level due to one of the following mechanisms: 1) a decrease in the proportion of cells that are able to express HSP 72; 2) a decrease in the level of HSP 72 expression by individual cells; or 3) some combination of both 1 and 2. In an effort to determine whether there is an age-related decrease in the proportion of cells with age that are able to express HSP 72, Heydari et al. used *in situ* hybridization to assess the ability of individual rat hepatocytes to express HSP 72 in response to heat stress (64). Their data indicated that essentially all of the cells from both young (6 mo. old) and senescent (26 mo. old) F-344 rats were able to express HSP 72. Thus, the age-related decrease in the induction of HSP 72 expression is not due an accumulation of cells that do not respond to heat stress.

Hence, by elimination, the age-related decline in HSP 72 expression is likely due to a decrease in the level of HSP 72 expression by individual cells.

There is growing speculation that the age-related attenuation in HSP 72 expression following heat or other stresses in several cell types is due to a reduced transcription of the gene (reviewed in 67). Specifically, studies using hepatocytes from rats (64) and cultured human diploid fibroblasts (23) have provided evidence that the age-related decline in HSP72 expression involves reduced binding of HSF1 to DNA. Importantly, the magnitude of change in DNA binding activity is consistent with the observed reduction in HSP72 expression. Together, these data suggest that the reduced ability of cells from old animals to express HSP72 in response to stress is due to a reduction in HSF 1 binding to the promoter region of the HSP72 gene. Hence, the key question becomes, "Why there is less HSF1 binding activity in aged cells?" At least two potential explanations exist. One possibility is that HSF1 protein levels decline with age. While this does not appear to be the case in both hepatocyctes and adrenal tissue (44,144), it is unknown if aging results in a reduced HSF1 protein levels in the myocardium. Our experiments addressed this issue.

Aging and Myocardial I-R Injury

Aging is associated with a progressive decline in most physiological functions; this is manifested by a decreased ability to maintain homeostasis when confronted with stress. The cardiovascular system is particularly vulnerable to aging-induced changes as aging constitutes a major risk for the development of cardiovascular diseases as the incidence of atherosclerosis, stroke, and myocardial infarction all increase markedly with advancing age (166). Importantly, aging results in a decreased tolerance to myocardial I-R injury. For example, elderly patients sustain a greater morbidity and mortality rate after myocardial infarction (MI) compared to younger patients (90). Further, numerous animal experiments suggest that aging directly increases the magnitude of myocardial I-R-induced injury in the senescent heart. For example, a recent *in vitro* experiment by Lesnefsky et al. demonstrated that senescent F-344 rat hearts sustain greater myocardial damage during an I-R insult compared to hearts from young/adult animals (91). Other investigations have reported similar findings (74,91,120).

The mechanism to explain the age-related decrease in myocardial tolerance to I-R remains unknown. Since I-R injury is associated with oxidative stress, it is possible that aging is associated with a reduced myocardial antioxidant capacity (77,113). The explanation for the lack of consistency in the literature is unclear and additional studies are required to resolve this issue.

Another potential explanation for an age-related decrease in myocardial tolerance to I-R could be due to the decline in the ability of the heart to activate heat shock protein expression (67,173). Indeed, since heat shock proteins protect cardiomyocytes during periods of ischemia, the inability of aged hearts to express heat shock proteins during transient periods of stress could contribute to the increased morbidity and mortality observed in the elderly.

Summary of Literature Review

One of the best understood cellular response to stress is the induction of heat shock proteins. HSPs are the products of highly conserved genes that are rapidly transcribed and translated in cells and tissues exposed to variety of stresses, including heat, oxidants, chemicals, and hormones. Since the optimal functioning and maintenance of any cell is explicitly dependent upon the presence of correctly folded proteins, chaperoning function of HSPs and particularly the 70 kDa family of HSPs are very important. One of the prominent member of 70 kDa family of HSPs is HSP72. The synthesis of HSP72 is usually restricted to the cell experiencing stress therefore is often referred to as inducible form of HSP70. Induction of HSP72 has been shown to enhance recovery from acute cellular injury and protect the cell from subsequent injury. We and others have shown that the physiological stress created by exercise training is capable of inducing HSP72 in the variety of tissues, including myocardium of the mammals.

Aged hearts are much more vulnerable to acute cardiovascular stress, and more susceptible to I-R injury than are young hearts. The inability of aging myocardium to respond to acute stress, may be explained, in part, by an alteration in the ability of cells to express HSP72 in response to acute stress. Indeed, our preliminary studies demonstrated that aged myocardium express less HSP72 compared with young myocardium after 10 weeks of endurance training. Currently the mechanism to explain the aged related attenuation in the HSP72 induction in old myocardium is unknown.

There is growing speculation that the age-related attenuation in HSP 72 expression following heat or other stresses in several cell types is due to a reduced transcription of the gene. Studies suggest that the reduced ability of cells from old animals to express HSP72 in response to stress is due to a reduction in HSF 1 binding to the promoter region of the HSP72 gene. To date, there are no published reports regarding the effects of aging on exercise-induced levels of HSP72 gene activation in the heart. By using intact animals and employ the tools of molecular biology (e.g., Gel mobility shift assay, and Western analysis), our experiments addressed this issue.

METHODS AND PROCEDURES

Animals and Experimental Design

This project was approved by the University of Florida Institutional Animal Care and Use Committee and followed the guidelines for animal use established by the American Physiological Society. Twenty two six 5-6 month (young/adult) and twenty two 23-24 months (old) old male Fisher-344 rats were employed in these experiments. Throughout the experimental period, animals were maintained on a 12-hour dark-light reverse cycle, individually housed at 25°C and 50% relative humidity, and provided food and water *ad libitum*. Upon arrival from the vendor, animals underwent a 1-week accommodation period and were then assigned to one of the following experimental groups:

Group 1) Young/adult control (n=6 rats)

Group 2) Old control (n=6 rats)

Group 3) Young/adult exercise training (n=8 rats)

Group 4) Old exercise training (n=8 rats)

Group 5) Young/adult heat stress (n=8)

Group 6) Old heat stress (n=8)

Experimental Protocols

Exercise Training Protocol

Animals assigned to an exercise training group were gradually introduced to treadmill running during a 7-day period. During this time, both treadmill speed and running time were gradually increased so that at the end of the week, animals were able to run continuously for 30 minutes at ~75% VO_2 max. Following this 7-day familiarization period, animals then performed five consecutive days of progressive exercise training. During these five days, training intensity was maintained at approximately 75%VO_2max, while the exercise duration was increased 10 minutes per day until reaching 60 minutes on the fourth day. This protocol ensured that all animals completed two consecutive days of 60 minutes of running (table 1). This training intensity and duration was chosen based on our preliminary experiments which indicated that this exercise protocol results in a significant increase in myocardial levels of HSP72 in young/adult rats. During the exercise training period, mild electrical shocks were used sparingly to motivate the animals to run. To standardize the stress of handling, control animals were placed daily on a non-moving treadmill.

Table 1. Summary of daily exercise training duration. Note, throughout the training animals ran on a motorized treadmill at~75% VO_2 max.

Exercise groups	Training duration (min)				
	Day 1	Day 2	Day 3	Day 4	Day 5
Group 3 (5-6-mo. old)	30	40	50	60	60
Group 4 (23-24-mo. old)	30	40	50	60	60

Heat Stress Protocol

Since whole body heat stress has been shown to result in binding of HSF to HSE and induction of both HSP72 mRNA and HSP72 protein, heat stress was incorporated into the experimental design as a positive control to compare with exercise training. Animals subjected to heat stress were anesthetized with pentobarbital sodium (35 mg/kg intraperitoneal) and placed on a heating pad set at 45°C. The animals remained on the heating pad until the rectal temperature reached 41.2°C; this temperature maintained for an additional 15 minutes. After 15-minutes of heat stress, the animals were administered 10-15 ml of water. The rationale for choosing 41.2°C temperature was that old rats can tolerate this type of stress and respond by significant increases in the expression of HSP72 in the myocardium (108).

Justification for Animal Model

There are very few animal models available to study aging. The National Institute of Aging has established rat and mouse colonies specifically for aging (54). Of these, the Fischer-344 rat was chosen because: 1) this animal has a relatively short life span with senescence attained at 24 months; 2) extensive background data are available; 3) this stock and strain has been characterized under well-defined environmental and genetic conditions with respect to age-associated changes; and 4) old rats are available from a reliable commercial breeder who maintains a barrier-reared aging colony of Fischer-344 rats under the close supervision of the National Institute of Aging. Since there is no evidence of a gender difference in the expression of myocardial HSP72, we arbitrarily chose male animals for our experiments.

Experimental Procedures

Tissue Removal

Animals were killed with an I.P injection of 90mg/kg phentobarbital and hearts were quickly removed. The left ventricles were then separated into sections, frozen in liquid nitrogen, and stored at –80°C until assay. Rats with documented pathology at the time of death were not included in the data analysis. Because the time to reach peak levels of HSP72 and HSF1 binding activity (binding of HSF1 to HSE) differ following stress, left ventricles from both heat stressed animals were removed at differing time periods. It is known that HSF is in the active state only for a short period (i.e. 10-60 minutes) after stress. On the other hand, accumulation of peak levels of HSP72 occurs within 24 hours following exposure to the stress. Therefore, subset of heat stressed animals (4 from each group) were killed immediately following heat stress and subset of heat stressed animals (4 from each group) were killed 24 hours after heat stress. Since animals have completed 60 minutes running in last two consecutive days of the exercise session, exercised animals were sacrificed immediately following last 60 minutes of treadmill running.

Hence, we performed Western analysis for HSP72, HSP73 and HSF1 on heart samples collected 24 hours following heat stress and 24 hours following 60 minutes of exercise training while we evaluated HSF-HSE binding (Gel mobility shift assay), immediately after heat stress and 60 minutes of treadmill running.

Preparation of Protein Extract

Portions of the left ventricle were thawed and homogenized in 5 volumes of extraction buffer (25% glycerol, 0.42 M NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA (pH 8.0), 20mM HEPES (pH 7.9), 0.5 mM DTT, 0.5 mM phenylmethylsulfonylfluoride) at 4°C at 5000 rpm using a mechanical homogenizer. Tissue homogenates were centrifuged at 15,000xg for 20 min in an Eppendorf centrifuge. After determining the protein concentration of the supernatant using the Bradford method (14), the supernatant was stored at –80°C and subsequently used for HSP72, HSP73, and HSF1 Western blotting and Gel mobility shift assays.

Polyacrylamide Gel Electrophoresis and Western Blotting

Protein extracts from control, heat stressed, and exercised rat hearts were mixed with Laemmli sample buffer to bring the concentration 2.5mg/ml. This mixture was then boiled for 4 minutes and separated on 12% of polyacrylamide gel electrophoresis (60µg protein). Proteins were then transferred to nitrocellulose membrane (0.45µm thick) using the Bio-Rad semi dry transfer system. Membranes were incubated with 3% BSA in PBS for 2 hours, and then with HSP72, and HSP73 (StressGen) specific monoclonal antibodies conjugated with alkaline phosphatase, and HSF1 polyclonal antirabbit antibody for 2 hours (StressGen). The membranes incubated with HSP72 and HSP73 antibodies were then reacted with bromochloroindolyl phosphate-nitroblue tetrazolium substrate. Membranes incubated with HSF1 antibodies were reacted with antirabbit secondary antibody conjugated with alkaline phosphatase for 2 hours. Visualization of HSF1 proteins was obtained by incubating membranes with bromochloroindolyl phosphate-nitroblue tetrazolium substrate. Quantification of the bands from the immunoblots was performed using computerized densitometry. Average intensities of HSP72, HSP73 and HSF1 bands from control young/adult hearts were compared against all other experimental groups.

Gel Mobility Shift Assay

Protein extracts (50μg) from control, heat stressed, and exercised rat hearts were incubated with an ^{32}P-labeled self-complementary ideal HSE oligonucleotide (5′-TCTAGAAGCTTCTAGAAGCTTCT-3′) in binding buffer (10% glycerol, 50mM NaCl, 1.0 mM EDTA (ph 8.0), 20 mM Tris (pH 8.0), 1.0 mM DTT, 0.3 mg/ml BSA) with 0.065 ng (50,000cpm) of ^{32}P labelled oligonucleotide and 5.0 μg poly (dI dC) for 20 min at room temperature. To determine the specificity of binding, reaction lysates were incubated in the presence of competing unlabeled HSE (10, 100, 200 and 300 fold excess), or in the presence of 200 fold excess unlabeled AP1 or SP1 oligonucleotides. Incubation reactions were fractionated by 4.5% nondenaturing polyacrylamide gel electrophoresis for 2-3 hours. Gels were then dried and analyzed after overnight exposure to phosphoscreen.

Statistical Analysis

Comparisons between experimental groups (young/adult vs. old) for each dependent variable data were made by factorial analysis of variance (ANOVA). When indicated, a Tukey's post hoc test was performed. Significance was established at $p<0.05$.

RESULTS

Morphometric Characteristics

Mean (±SEM) body mass and heart weights of the animals for both age groups are presented in table 2. Within the same age group, body mass, heart weight and heart weight to body mass ratios did not differ between the experimental groups. However, compared to young/adult rats, old animals demonstrated a greater body mass ($p<0.05$) and heart weight ($p<0.05$).

Table 2. Morphometric characteristics of young/adult and F-344 rats. Values are expressed as means ±SEM. Statistically significant differences (old vs. young/adult at $p<0.05$) are indicated (*).

	Groups	Control	Trained	Heat Stressed
Body weight (g)	Young/Adult	353.3±5.5	350.3±4.5	355.2±4.2
	Old	422.7±8.6*	424.7±9.5*	429.1±7.5*
Heart weight (mg)	Young/Adult	958±17	955±19	960±21
	Old	1141.3±27*	1148.2±23*	1150.2±30*
Heart weight (mg)/Body weight(g) ratio	Young/Adult	2.71±0.05	2.72±0.06	2.70±0.004
	Old	2.70±0.04	2.71±0.06	2.68±0.06

Western Blotting for HSP72

Figure 1 illustrates a typical Western blot to determine myocardial HSP72 levels in the control, heat stressed and trained groups from both young/adult and old animals.

Figure 2 presents a comparison of the mean myocardial levels of HSP72 in all groups. Control animals from both young/adult and old animals expressed similar basal levels of myocardial HSP72. Both heat stress and exercise training resulted in a significant induction of HSP72 in the myocardium of both young/adult and old rats ($p<0.05$). This increase was significantly higher in the exercise training groups compared with the heat stress groups (200% vs. 350% in young/adult rats and 150% vs. 300% in old rats, respectively; $p<0.05$). Compared to young/adult animals, old animals expressed significantly less myocardial HSP72 following heat stress and training (200% vs. 150% increase in heat stressed groups, and 350% vs. 300% in the exercise trained groups, respectively; $p<0.05$).

Western Blotting for HSP73

Figure 3 illustrates Western blotting of HSP73 in the control, heat stressed and trained groups from both young/adult and old animals. Figure 4 contains a comparison of the myocardial levels of HSP73 in all groups. Control animals from both young/adult and old animals expressed similar basal levels of HSP73. Neither heat stress nor exercise training increased the myocardial levels of HSP73 in young/adult or old animals ($p>0.05$).

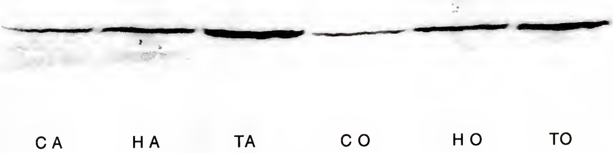

Figure 1. A Representative Western blotting for HSP72.

Sixty micrograms of protein from each sample were separated on a 12% SDS-polyacrylamide gel via electrophoresis. Samples were then transferred to nitrocellulose membranes and incubated with the HSP72 antibody. CA= control adult; CO= control old; TA= trained adult; TO= trained old; HA= heat stressed adult; and HO= heat stressed old rats

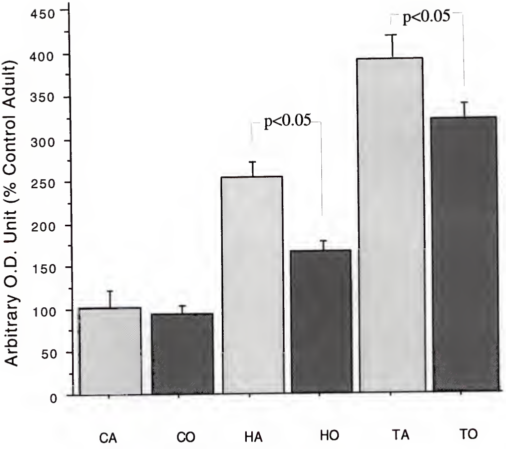

Figure 2. HSP72 expression from the myocardium of adult and old animals following exercise training and heat stress.

Values are mean±SEM of percentage control adult rats. CA= control adult; CO= control old; TA= trained adult; TO= trained old; HA= heat stressed adult; and HO= heat stressed old rats.

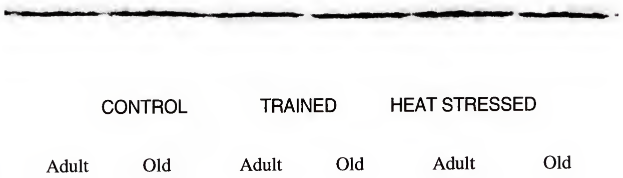

Figure 3. A Representative Western blotting for HSP73.

Sixty microgram of protein from each sample were sepArated on 12% SDS-polyacrylamide gel electrophoresis. Samples were then transferred to nitrocellulose membrane and incubated with HSP73 antibody.

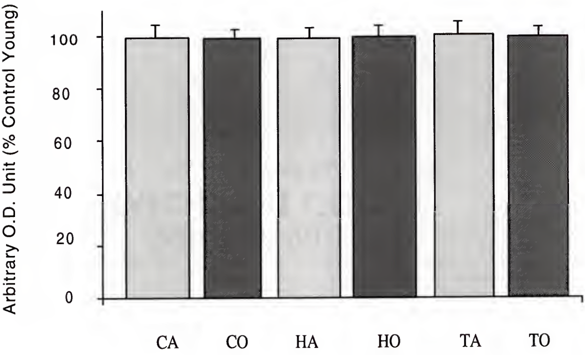

Figure 4. HSP73 expression from myocardium of adult and old animal following exercise training and heat stress.

Values are mean±SEM of percentage control adult rats. CA= control adult; CO= control old; TA= trained adult; TO= trained old; HA= heat stressed adult; and HO= heat stressed old rats.

Western Blotting for HSF1

Figure 5 illustrates Western blotting to determine the myocardial levels of HSF1 in the control, heat stressed and trained groups from both young/adult and old animals. Figure 6 contains a comparison of the myocardial levels of HSF1 in all groups. Control animals from both young/adult and old animals expressed similar basal levels of HSF1. Neither heat stress nor exercise training increased the basal level of HSF1 in young/adult and old groups ($p>0.05$).

Gel Mobility Shift Assay for HSF-HSE Binding

Figure 7 and 8 illustrate HSF-HSE binding in the control, heat stressed and trained groups from both young/adult and old animals. Myocardial extracts from the majority of young/adult and old control (unstressed) rats revealed no HSF-HSE binding. Nonetheless, gels from one of the young/adult and old rats (1 out of 6 rats from each age group) demonstrated a small band shift indicating HSE-binding. Following both exercise and heat stress, HSF-HSE binding was detected in myocardial extracts from both young/adult and old rat animals. Although we observed a diminished level of myocardial HSP72 protein expression in old animals following both heat stress and exercise, HSF-HSE binding did not differ between young/adult and old animals following either stress.

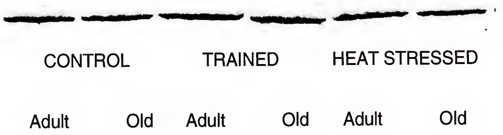

Figure 5. A Representative Western blotting for HSF1.

Sixty micrograms of protein from each sample were seperated on a 12% SDS-polyacrylamide gel via electrophoresis. Samples were then transferred to nitrocellulose membranes and incubated with the HSF1 antibody. CA= control adult; CO= control old; TA= trained adult; TO= trained old; HA= heat stressed adult; and HO= heat stressed old rats

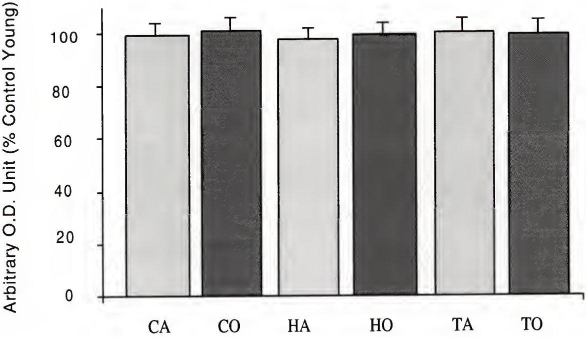

Figure 6. HSF1 expression from the myocardium of adult and old animals following exercise training and heat stress.

Values are mean±SEM of percentage control adult rats. CA= control adult; CO= control old; TA= trained adult; TO= trained old; HA= heat stressed adult; and HO= heat stressed old rats.

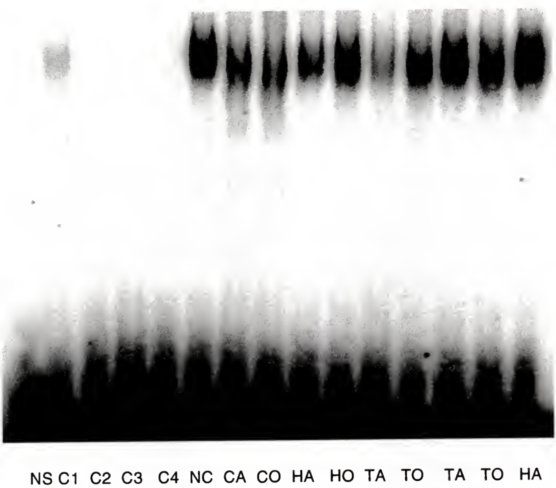

Figure 7. Analysis and specificity of HSE-binding activity of myocardial extracts.

Gel mobility shift assays were performed using whole tissue extracts (50 microgram protein from each lane). NP = no protein, mobility of unbound labeled HSE oligonucleotide; C1= 10 fold excess unlabeled HSE oligonucleotide; C2= 100 fold excess unlabeled HSE oligonucleotide; C3= 200 fold excess unlabeled HSE oligonucleotide; C4= 300 fold excess HSE oligonucleotide; NC= 200 fold excess unlabeled SP1 oligonucleotide; CA= control young/adult; CO= control old; TA= training young/adult; TO = training old; HA= heat stressed young/old; HO = heat stressed old .

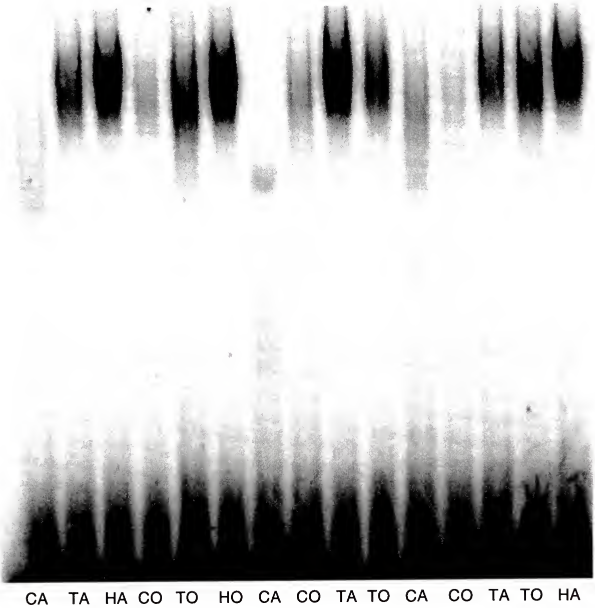

Figure 8. Analysis of HSE-binding activity in myocardial extracts.

Gel mobility shift assays were performed using whole tissue extracts (50 microgram protein from each lane). CA= control young/adult; CO= control old; TA= training young/adult; TO = training old; HA= heat stressed young/old; HO = heat stressed old .

DISCUSSION

Overview of Principle Findings

These experiments confirm that aging is associated with a reduced ability to express myocardial HSP72 in response to exercise stress. The primary purpose of this study was to test two related hypotheses relative to the mechanism responsible for this age-related decrease in HSP induction in the senescent myocardium. First, it was hypothesized that aging is associated with a decreased myocardial levels of HSF1. Secondly, we hypothesized that compared to young/adult animals, senescent animals would experience less exercise-induced binding of HSF1 to the HSE in the promoter region of the myocardial HSP72 gene. It follows that a reduction in HSF1-HSE binding would result in less activation of the gene and therefore less expression of HSP72.

Our data do not support these hypotheses. Indeed, our results indicate that myocardial levels of HSF1 do not differ between young/adult and old animals. Further, both young/adult and animals experienced similar levels of myocardial HSF1-HSE binding following both exercise and heat stress.

Aging and HSP72 Induction Following Exercise Training or Heat Stress

The current data confirm previous reports that both tissues and primary cell cultures derived from old animals have a reduced ability to express HSP72 in response to heat stress (11,12,43,62,64,99,103,108), Further, the current experiments indicate that aging is associated with a reduced ability to express myocardial HSP72 following exercise stress. This could be physiologically significant because HSP72 has been shown to protect cells against a variety of stresses (5,15,72, 82, 95, 102,111,137) including myocardial I-R injury (25,27,32,34,40,111,138,165). This reduced ability of aged hearts to express HSP72 may explain, at least in part, why senescent hearts are more vulnerable to acute cardiovascular stress, and more susceptible to ischemia- reperfusion injury than are young hearts (74).

It is unknown if the level of induction of HSP72 in the current study is large enough to protect the aged myocardium from I-R injury. In this regard, Locke and Tanguay evaluated myocardial performance following an *in vitro* I-R procedure after heat stress in both old and adult animals (108). In these experiments, heat stress resulted in a significant increase in myocardial HSP72 levels in both young and old animals; however, compared to young animals, myocardial HSP72 levels were lower in the old animals. The elevation in cardiac HSP 72 following heat stress was associated with reduced I-R-induced myocardial injury in the young animals. In contrast, heat stress in the old animals did not provide myocardial protection against the I-R insult. Since the significant but reduced HSP72 induction in old myocardium did not provide protection against myocardial I-R injury, these authors speculated that a "critical level of HSP72" may be required to provide protection (108). Additional studies are required to determine if exercise-induced elevation in myocardial HSP72 is sufficient to provide protection against I-R injury in aged animals.

In previous studies we have observed a 500% increase in myocardial HSP72 levels in young/adult rats following 12 weeks of endurance exercise training (33,34,138).

In this current study, the exercise-induced expression of HSP72 in the young/adult myocardium was of lower magnitude compared to the aforementioned exercise studies. This between-study difference in exercise-induced myocardial HSP72 could due to one or more factors including the duration of the exercise training (i.e. 5 days vs. 12 weeks) and/or sex and strain differences (i.e. current study employed male Fisher-344 rats whereas female Sprague Dawley rats were used in our previous studies).

Aging and HSP73 Levels Following Exercise Training and Heat Stress

Although this current study focused on the investigation of the inducible form of 70-kDa HSPs, HSP72 , we also examined the constitutive form of the 70-kDa HSP family, HSP73 in the myocardium of young/adult and old rats following exercise training. Our data indicate that the basal level of HSP73 did not change following exercise training or heat stress (see figures 3 and 4). Similar results in rat hepatocytes have been reported by others (175).

In contrast, we previously observed that high intensity endurance training (75%VO_2max, >60 min/day, 10 weeks of total duration), results in a significant increase in HSP73 levels in the adrenals but not in myocardium of rats (33,34). A definitive explanation for these observations is not available. However, it has been postulated that the adrenal gland is very sensitive to the types of stresses that induce an HSP gene response (12). Therefore, it is possible that the physiologic stress created by acute exercise training is great enough to induce the expression of HSP73 in the adrenal gland but is not sufficient to induce an HSP73 response in rat myocardium.

Aging and HSF1 Levels Following Exercise Training and Heat Stress

In mammals, the expression of HSF1 is constitutive and does not appear to be heat inducible (147,148). Induction of HSP genes is achieved by activating preexisting HSF1, which then binds to the HSE and activates transcription. At present, no information is available related to the possible effects of exercise and aging on the HSF1 level and how this may effect the induction process. Since HSF1 is the transcription factor for HSP genes, there would be two possible changes in the regulation and function of HSF1 as a basis for attenuated heat shock response in aging myocardium following exercise training: 1) a decrease in the expression and basal level of HSF1; or 2) disconcerted activation mechanism of HSF1. Therefore, we included the measurement of HSF1 in this study. Our data demonstrated that both old and young/adult unstressed control animals expressed similar basal levels of myocardial HSF1. Further, exercise training and heat stress did not effect the constitutive level of HSF1 in young/adult and old animals (see figures 5 and 6).

Aging and HSF-HSE Binding Following Exercise Training and Heat Stress

In the current experiments we hypothesized that attenuation of HSP72 induction in old myocardium following exercise training is due to reduced binding of HSF to HSE; therefore, reduced gene activation. Nonetheless, our data do not support this hypothesis, since the myocardial extracts from old stressed animals did not demonstrate a reduced amount of HSF-HSE shift on our gel mobility shift assay.

Activation of HSF is a multistep process that can be divided into four phases: 1) oligomerization of inactive monomers to trimers; 2) nuclear localization; 3) DNA binding at the promoter; and 4) final modulation of HSF1 leading to transcriptional competence. Since the basal levels of HSF1 does not differ between young adult an old animals, it appears that one of the aforementioned step toward activation of HSF1 could be affected, and therefore, responsible for the attenuated level of final gene product following exercise with aging. Alternatively, the steps leading to mRNA after transcriptional competency and/or translation could also be affected with aging. A brief discussion of the steps required to achieve overall transcriptional competency and an interpretation of our findings follows.

Oligomerization.

In unstressed cells, HSF1 is located both in the cytoplasm and in the nucleus as an inactive monomeric state. After exposure to protein damaging stresses, HSF1 undergoes a conversion to a trimeric state and acquires DNA binding activity (6). Interactions between HSF1 and one or more HSPs as well as other proteins have been implicated in maintaining the folded, or inactive, state (21,141,169).

Early models suggested that HSP72 primarily regulated HSF activity (1,24,126). However, new evidence indicates that HSP90 may be a key molecule regulating trimerization and activation of DNA binding of HSF1 (180). *In vitro* experiments have shown that depletion or inactivation of HSP90 in cell free extracts results in the activation of HSF1-DNA binding. This revised model for HSF1 activation now has HSP90 (or a multichaperone complex containing HSP90 and small amounts of HSP72) bound in equilibrium to HSF1 under nonstressed conditions and maintaining the folded inactive state (6,7,141,179-180). During cellular stress, free pools of HSP90and HSP72 are depleted by interactions with denatured cellular proteins resulting in the unfolding of

HSF1; this permits trimerization. Therefore, increased levels of interactions between HSF1 and HSP72, HSP90 and/or some unknown molecule(s) in aging animals may be responsible for less induction of HSP72 following exercise training.

The *in vivo* HSF1-HSE binding requires oligomerization, nuclear localization, and binding of HSF to a specific promoter region on HSP gene. Analysis of our *in vitro* gel mobility shift assays indicates that old and young/adult animals have similar levels of HSF1-HSE binding immediately after running exercise. Therefore, since oligomerization is required for HSF1-HSE binding, these results suggest that there is not an age-related compromise at the oligomerization step of HSP72 expression.

Nuclear localization and 3) DNA binding.

The second and third steps in HSF activation involve nuclear localization and DNA binding. In *Drosophila*, HSF1 is localized to the nucleus (172). However, in mammalian cells HSF1 is distributed throughout the cytoplasm and nucleus in the absence of stress and upon exposure to heat shock, HSF1 moves from the cytoplasm to the nucleus where it binds to the heat shock element on the promoter as a trimer (148). Therefore, additional regulation of HSF by compartmentalization may play a role in difference HSP72 induction reported in the current study. Any reduction in nuclear localization and DNA binding of HSF1 in old animals following exercise would account for the attenuated HSP72 response. In that respect, reduced binding of HSF1 to DNA has been shown in hepatocytes isolated from old rats (60,175), aging human diploid fibroblasts (18,103), and myocardium from whole body heat stressed aged rats (108). As opposed to aforementioned first two studies, the current experiments used whole tissue extracts instead of nuclear extracts for HSE-binding assays. Therefore, our results do not exclude the possibility of reduced nuclear localization, or reduced trimerization of HSF monomers, which are already located in the nuclei. In that respect, our HSF protein measurements do not reflect the level of HSF in the nuclei but the overall level of HSF in the cell.

Final modulation of HSF:

The last step in the activation of HSF1 requires molecular modification of HSF; this event results in the acquisition of transcriptional competence. To date, limited information is available regarding the molecular mechanism(s) responsible for the activation of transcriptional competence; however, a significant correlation exists between phosphorylation of HSF1 and transcriptional activity (23). Further, since HSF1 binding to DNA precedes the phosphorylation step , it appears that HSF1-DNA binding and transcriptional activation is induced by separate mechanisms (23). Although HSF from *Drosophila* and mammalian cells was shown to have latent activity which was activated for DNA binding and transcriptional activity upon heat shock (127), yeast HSF bound to DNA constitutively and heat shock was required to activate transcription, which correlated with the inducible phosphorylation of HSF (156). Less phosphorylation of HSF1 in aged myocardium also could result in less transcription of HSP72 gene and less induction of HSP72 following exercise training. In that respect, evidence exist that oxidative stress rapidly activates the heat shock transcription factor but fails to increase the levels of HSP72 mRNA and protein (17). In this report, the authors raised the possibility that oxidants may cause only the initial oligomerization step but not the subsequent phosphorylation of HSF1, since the nuclear extracts of H_2O_2 treated NIH-3T3 cells expressed as much HSE-binding as heat stressed NIH-3T3 cells (17). Activation of the HSF binding activity but failure to cause accumulation of HSP72 mRNA has been reported in permeabilized cells which have been deprived of ATP or treated with protein kinase inhibitor, genistein (140), or in MEL cells, in which the HSF is activated by heat but is nor phosphorylated (58).

Another example for the role of phosphorylation for HSF activation is the effect of flavenoid compound quercetin. Quercetin was shown to interfere with the activation of HSF1 DNA binding activity by inhibiting heat stress induced phosphorylation of HSF1 (68).

Although we did not determine the phosphorylation status of myocardial HSF1 in our exercised animals, similar levels of HSE-binding with the attenuated induction of HSP72 in old myocardium may be the result of reduced phosphorylation of HSF1 in old animals after stress. In this regard, a recent report indicates that salicylate activation of DNA binding by the HSF was comparable to activation attained during heat shock, but it did not induce heat shock gene transcription, even though salicylate activated HSF-DNA binding activity was nuclear localized and bound *in vivo* to the heat shock elements upstream of the HSP72 gene (78). These results indicate that activation of the heat shock transcriptional response is a multistep process and that oligomerization, nuclear localization, and binding to HSE *in vivo* does not necessarily lead to transcriptional competency.

In the present experiments, the myocardial levels of HSF1 and HSF1-HSE binding (following heat and exercise) did not differ between young/adult and old animals. Therefore, it appears that the age-related differences in exercise-induced myocardial HSP72 expression is due to steps other than oligomerization and DNA binding. In the current experiments, we did not measure the HSP72 mRNA levels or *in vivo* transcription rates of HSP72. Hence, we do not know if there was an attenuated transcriptional competency in exercised old animals in this study. Clearly, attenuated transcriptional competency could explain the reduced HSP72 response in old rats without effecting the binding of HSF to HSE following exercise.

Summary and Conclusions

The present study was designed determine if the age-related reduction in exercise-induced expression of myocardial HSP72 is due to decreased activation of the HSP72 gene. Specifically, these experiments tested two primary hypotheses: 1) aging is associated with decreased myocardial levels of HSF1; and 2) compared to young/adult animals, senescent animals experience less exercise-induced binding of HSF1 to the HSE in the promoter region of the myocardial HSP72 gene. These postulates were tested by examining the *in vitro* binding of heat shock transcription factor HSF1 on the promoter region of the HSP 72 gene, along with the expression of HSP72, HSP73 and HSF1 in old (23-24 month old) and young/adult (5-6 month old) hearts following both exercise training and whole body heat stress.

Our results confirm that aging is associated with a reduced ability to express myocardial HSP72 in response to exercise or heat stress. Theoretically, decreased steady state levels of HSF1 or reduced binding ability of HSF1 to HSE could be account for this age-related decline in HSP72 induction. Nonetheless, our results clearly indicate that the myocardial levels of HSF1 do not differ between young/adult and old animals. Further, our experiments reveal that the attenuated expression of HSP72 in the old myocardium was not the result of a reduction in HSF1 binding to the HSE on the promoter region of the HSP72 gene. Therefore, our results do not support our experimental hypotheses.

In summary, the age-reduced ability to express myocardial HSP72 in response to exercise or heat stress is not due to lower myocardial levels of HSF1 or a reduction in HSF1 binding to DNA on the HSP72 gene. By elimination, we conclude that the age-related depression in myocardial HSP72 levels following exercise or heat stress are due to one or more of the following factors: 1) reduced acquisition of transcriptional competency resulting in less HSP72 mRNA; 2) shortened half-life of HSP72 mRNA; 3) impaired translation resulting in reduced synthesis of the HSP72 protein; and/or 4) reduced half-life of HSP72 protein. Clearly additional experiments to resolve this issue are warranted.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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